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Biotransformation of dietary inorganic arsenic in a freshwater fish Carassius auratus and the unique association between arsenic dimethylation and oxidative damage

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Biotransformation of Dietary Inorganic Arsenic in a Freshwater Fish *Carassius auratus* and the Unique Association between Arsenic Dimethylation and Oxidative Damage

Di Cui (Conceptualization) (Methodology) (Validation) (Investigation) (Supervision) (Project administration) (Data curation) (Writing - original draft) (Writing - review and editing), Peng Zhang (Writing - review and editing) (Validation) (Funding acquisition) (Formal analysis) (Visualization) (Software), Haipu Li (Resources) (Supervision) (Funding acquisition) (Writing - review and editing), Zhaoxue Zhang (Methodology) (Writing - review and editing) (Data curation), Wenbao Luo (Investigation) (Validation), Zhaoguang Yang (Resources) (Supervision) (Funding acquisition) (Visualization)

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Biotransformation of Dietary Inorganic Arsenic in a Freshwater Fish *Carassius auratus* and the Unique Association between Arsenic Dimethylation and Oxidative Damage

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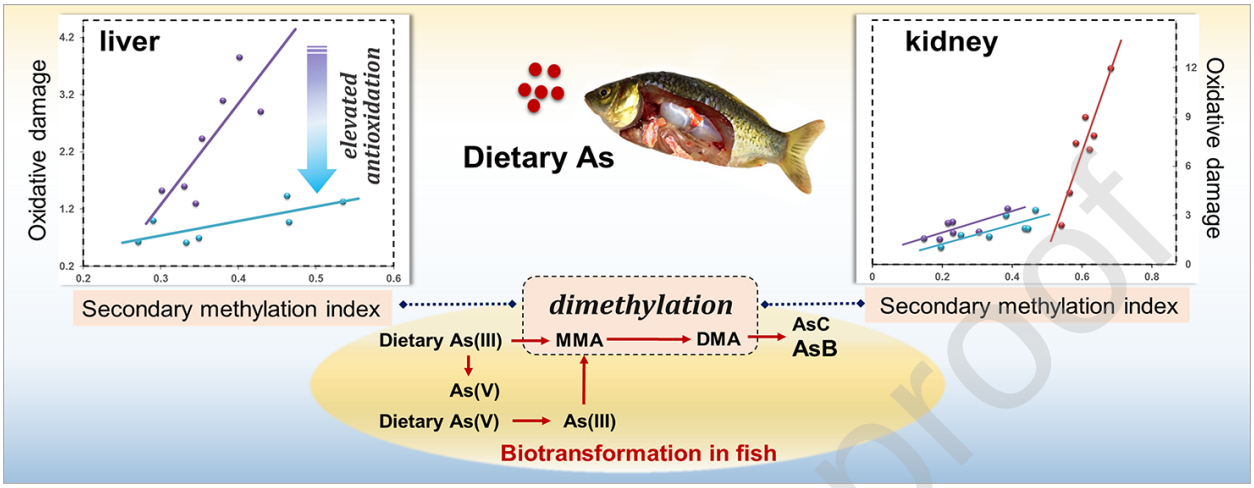
Graphical abstract



Highlights

- •Parallel conversion between arsenite and arsenate occurred in freshwater fish.
- •The arsenic dimethylation activity induced oxidative damage.
- •Acclimated physiology occurred after dietborne arsenic exposure to resist toxicity.
- •Arsenobetaine became predominant in freshwater fish after dietborne arsenic exposure.

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ABSTRACT: The metabolic process and toxicity mechanism of dietary inorganic arsenic (iAs) in freshwater fish remain unclear to date. The present study conducted two iAs [arsenate (As(V)) and arsenite (As(III))] dietary exposures in freshwater fish crucian carp (*Carassius auratus*). The fish were fed on As supplemented artificial diets at nominal concentrations of 50 and 100 μg As(III) or As(V) g^{-1} (dry weight) for 10 d and 20 d. We found that liver, kidney, spleen and intestine accumulate more As in As(V) feeding group than that in As(III), while the total As level in muscle were similar between As(V) and As(III) group at the end of exposure. Reduction of As(V) to As(III) and oxidation of As(III) to As(V) occurred in fish fed with As(V) and As(III), respectively, indicating that toxicity of iAs were likely elevated or reduced when iAs was absorbed by fish before entering into human body through diet. Biomethylation to monomethylarsonic acid and dimethylarsinic acid and transformation to arsenocholine and arsenobetaine were also found in the fish. The linear regression analysis showed a positive correlation between secondary methylation index and the malondialdehyde content in tissues, highlighting the vital role of arsenic dimethylation in the oxidative damages in fish.

KEYWORDS: Arsenic; Bioaccumulation; Biotransformation; Oxidative stress; Freshwater fish

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1. Introduction

Arsenic (As) is a prevalent environmental toxicant with human health implications and is ranked the first on 2017 Priority List of Hazardous Substances (https://www.atsdr.cdc.gov/spl/index.html). The release of As into aquatic environment, as a result of the mining, smelting of metals, and the burning of fossil fuels, has led to high levels of As in sediments (Hong et al., 2018; Pedlar et al., 2002). The As in sediments is biologically available to benthic fish through diet. It has been reported that, As bioaccumulation in benthic fish was positively correlated with As concentration in sediments, however, with no significant association with the soluble As in water (Hong et al., 2018; Zhang et al., 2018). Although an important way of As uptake by benthic fish is dietary, the majority of researches on As toxicity and metabolism in fish have investigated the effects associated with uptake of waterborne As across the gills. Experiments that investigate the toxicological effects and metabolism pathway of As in fish exposed by the dietborne route of uptake are needed in order to determine parameters that could be used as reliable and sensitive indicators of As toxicity in environmental monitoring programs.

Inorganic arsenic (iAs), including arsenate(As(V)) and arsenite(As(III)), are the main As species in sediment (Cullen and Reimer, 2010). This will undoubtedly increase the potential of benthic fish exposure to iAs by dietary pathway. Organisms have systems for iAs biotransformation, while the effectiveness of the detoxification would depends on the relative production of toxic As species compared with the non-toxic As species (Rahman and Hassler, 2014). Most laboratory experiments on As biotransformation in fish, however, were performed via waterborne exposure, with the exception of only a few dietborne experiments using marine fish. For example, the conversion of iAs to arsenobetaine (AsB) has been reported in marine rabbitfish (*Siganus fuscescens*) and grunts (*Terapon jarbua*) (Zhang et al., 2016a; Zhang et al., 2016b). The

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transformation product of iAs in freshwater fish varies in different studies. For example, As mainly presented as trimethylarsenate and monomethylarsonic acid (MMA) in tilapia (*Tilapia mossambica*) and medaka (*Oryzias latipes*), respectively, after exposed to As(V) and As(III) for 7 d (Suhendrayatna et al., 2002a; Suhendrayatna et al., 2002b). Bears et al. (2006) showed that dimethylarsinic acid (DMA) was the dominant As compound in *Fundulus heteroclitus* after 787 μg L^{-1} As exposure for 14 days. Most of these results from freshwater fish, however, were obtained in waterborne exposure studies. The specific metabolism pathway of dietary iAs in freshwater fish has yet to be fully understood and remains under debate.

Human exposure to iAs could affect multiple organ functions, resulting in various As-related diseases including cancers (Manthari et al., 2018; Signes-Pastor et al., 2008). The aetiopathogenesis of arsenotoxicosis is proposed to be linked to an imbalance between antioxidant and prooxidant homeostasis that results in oxidative stress (Watanabe and Hirano, 2013). Methylation of iAs has long been considered to be a detoxification process. However, recent data point to the fact that the trivalent intermediates in the methylation process in mammal, MMA(III) and DMA(III), are more active than iAs in terms of cytotoxicity, genotoxicity, and enzyme inhibition (Kuo et al., 2017). Therefore, understanding the iAs biotransformation processes in fish, especially the methylation process, and their association with toxic symptoms such as oxidative damage, are essential for assessing and predicting the toxicity of iAs. However, up to date, whether the methylation is to detoxify or potentiate arsenic toxicity remains an ongoing debate and need the study outcome.

Crucian carp (*Carassius auratus*) is a kind of food fish that is widely distributed in freshwaters throughout China and is considered as a very sensitive and suitable species for the study of biotransformation responses (Zhang et al., 2004). Due to its palatability, fast growth rate, and high

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nutritional quality, crucian carp is economically important in China; its yield accounts for 15% of the total fish productions in China (Deng et al., 2012). However, as a benthic-feeding fish species, crucian carp can easily ingest sediment As by dietary pathway. Therefore, revealing the behavior and toxicity of iAs in crucian carp is crucial for not only fish fishery but also human safety.

The objective of this study was to investigate the bioaccumulation and biotransformation of As(III) and As(V) in different tissues of freshwater fish crucian carp by dietborne exposure. At the same time, the levels of tissues oxidative stress induced by iAs were monitored. The link between the toxicity (oxidative damage) and iAs biotransformation was explored to comprehend the toxic effect of iAs intake by food in freshwater fish. To our knowledge, this have not been investigated previously through animal model with iAs dietborne exposure.

2. Materials and methods

2.1. *Fish and dietary inorganic arsenic exposure*

Crucian carp (*C. auratus*) (13.8–15.5 cm in length) were purchased from a fishery at Changsha, China. They were cultured in dechlorinated municipal water (24 °C) and fed artificial puffed pellet diet (obtained from Huize Biological Technology Co. Ltd, Hubei, China) at 1% of fish weight three times per day. Since crucian carp is benthic fish, acclimation is required to train them to feed the floating puffed pellet diets that were used in this study. Prior to experimentation, fish were acclimatized for 14 days to allow training on battle for the floating diet.

As(V) or As(III) was added to the artificial fish diets as an arsenic solution of arsenate or arsenite ($Na_2HAsO_4 \cdot 7H_2O$ or $NaAsO_2$, O2SI, USA), respectively, to attain two environmentally relevant concentrations of 50 and 100 μg g^{-1} (Chai et al., 2017). The artificial diet pellets were immersed within a definite amount of arsenic solution. Meanwhile, continuously rotating and

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flipping were needed to ensure the uniformity of the soak. Until the absorption was completed, for about five minutes, the pellets were lyophilized in a freeze dryer to constant weight. The diets were stored in sealed polyethylene bags at -20 °C until use.

100 crucian carps were exposed to five dietborne treatments (control, 50 μg g^{-1} As(III), 50 μg g^{-1} As(V), 100 μg g^{-1} As(III), 100 μg g^{-1} As(V)) with five 100-liter indoor aquaria (20 individuals per tank; 5-liter per fish). They were fed three times a day at 1% of fish weight for 10 d and 20 d. To prevent any As exposure from water, the uneaten diets were removed immediately and the tank water was changed every 24 hours. During exposure period, there was no significant difference in fish diet amounts among the groups. At the exposure termination of 10 d and 20 d, fish were famished for about 24 hours to allow the elimination of gut contents. Fishes (n = 7) in each treatment were then collected and the body surface was cleaned by washing with deionized water. The water on the surface was then blotted on clean tissues, and the weight and length of each individual fish were measured. Fish were anesthetized and euthanized and tissues including liver, kidney, spleen, intestine and the muscle of whole body were immediately collected. Parts of liver, kidney and spleen samples were washed in 0.15 M of cold KCl solution, immediately frozen in liquid nitrogen, and stored at -80 °C for biochemical analysis. The remaining parts of liver, kidney and spleen, and the total of intestine and muscle, were freeze dried (freeze drier, BIOCOOL, Lab-1A-50E, Beijing Boyikang Experimental Instrument Co., China) and homogenized for total arsenic and arsenic speciation analysis. The condition factor (CF = weight (g)/$length^3$ (cm)), kidney somatic index (KSI = kidney wet weight (g)/fish wet weight (g) × 100%), liver somatic index (LSI = liver wet weight (g)/fish wet weight (g) × 100%), and spleen somatic index (SSI = spleen wet weight (g)/fish wet weight (g) × 100%) were calculated for each individual fish.

Fish experiments were conducted according to the guidelines of the animal ethical committee

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for animal experimentation in China. All efforts were made to minimize fish suffering and the numbers of fish used.

2.2. *Total arsenic concentration*

About 0.04 - 0.2 g of samples were weighted and digested with a mixture of 2 mL H_2O_2 (30% wt, guaranteed reagent grade, Aladdin Reagent Co. Ltd., China) and 10 mL HNO_3 (70% wt, ⩾ 99.999% metal basis, Aladdin Reagent Co. Ltd., China) on a microwave digestion system (MDS-6G, Sineo Microwave Chemistry Technology Co. Ltd, Shanghai, China). The digestion followed as the procedure: 15 min to 120 °C, 15 min to 180 °C, and 30 min at 180 °C. After cooling, the samples were diluted to 25 mL with deionized water (18.2 MΩ cm, Direct-Q 3, Millipore SAS, France). Arsenic concentrations were analyzed by inductively coupled plasma-mass spectrometer (ICP-MS, Agilent 7700x, Japan). The standard solution was prepared by serial dilution from a stock solution (National Analysis Center for Nonferrous Metals and Electronic Materials, Beijing, China). A standard reference material (SRM, BCR-627 Tuna Tissue, Institute for Reference Materials and Measurements, Belgium) was also digested to estimate the performances of the digestion and analytical methods. In addition, we prepared an in-house reference sample by adding 5 μg/L As standard to low-arsenic fish tissues. The total As recovery rate was 88.6-103.9%. During the measurement, germanium was used to compensate for signal drifting and matrix suppression. Recovery from BCR-627 was 100.5±3.2%. The method blank was performed throughout the entire sample preparation for each batch. The concentrations of As were expressed as μg g^{-1} dry weight (dw). The As(V)-dosed diets and As(III)-dosed diets were also digested and the total As concentrations were shown in Table S1.

2.3. *Arsenic species analysis*

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The extraction and analysis of As species were processed according to the procedure optimized by Jia et al. (2018). Briefly, the dry sample powder were soaked in 1% HNO_3 and heated at 100 °C for 90 min on a microwave extraction system. The extracted solutions were diluted to 25 mL with deionized water and filtered through 0.22 μm syringe filters. Anion exchange high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS; HPLC, Agilent 1260, Tokyo, Japan) with gradient elution was adopted to separate and analyzed the arsenic species, including As(III), As(V), MMA, DMA, arsenocholine (AsC) and AsB. The operating parameters for HPLC and ICP-MS followed the description by Jia et al. (2018). Stock solutions of As(III) and As(V) were obtained from O2SI smart solutions (Charleston, SC, USA). Stock solutions of MMA and DMA were prepared from methylarsonic acid mono sodium salt hydrate and cacodylic acid sodium salt (Dr. Ehrenstorfer, Ausberg, Germany), respectively. Stock solutions of AsC and AsB were diluted from concentrated corresponding solutions provided by CRM/RM information center (Beijing, China). The SRM BCR-627 and spikes were utilized to confirm the analysis methods and extraction efficiency. BCR-627 tuna tissue contained DMA 1.25±0.19 μg g^{-1} (84.3±12.7% recovery, n = 5) and AsB 3.72±0.31 μg g^{-1} (95.1±7.9% recovery, n = 5). Because there was no SRM for fish muscle certified for other four As species (AsC, AsIII, MMA, or AsV), and there was no SRM for fish liver, kidney, spleen or intestine, the in-house reference samples were prepared by adding 3 μg/L As standard mixture to low-arsenic fish tissues. The recovery were 93.3-104.1% for AsB, 76.1-91.4% for AsC, 82.4-93.7% for MMA, 79.2-91.8% for DMA, 80.2-95.5% for AsV, and 78.6-93.4% for AsIII. The method detection limit (MDL), obtained according to the method of the U.S. Environmental Protection Agency (EPA) (2011) by seven replicate analyses of method blank, were 9, 3, 3, 1, 2 and 3 μg kg^{-1}, respectively, for As(V), As(III), DMA, MMA, AsC and AsB. For each batch of prepared samples, the method blank was performed

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throughout the entire extraction procedure. The arsenic speciation concentrations of control (basal) and As-dosed diets were also measured and the results were shown in Table S1.

2.4. *Biochemical analysis*

The liver, kidney, and spleen of fish were homogenized in chilled phosphate-buffered saline (PBS, PH 7.4, 0.01 M) using a tissue homogenizer (TL2010s, DHSBIO). The tissues homogenates were centrifuged at 9000 × g for 10 min at 4 °C. The resultant supernatants were adopted for analysis of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione (GSH) after suitable dilution, using the assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.5. *Statistical analysis*

The data processing was performed by utilizing SPSS version 19.0. Arithmetic mean and standard deviation (SD) in the tables and figures were calculated based on the results from analyses of 7 different fish samples in each test treatment. One-Way ANOVA followed by a least significant difference (LSD) test was used to assess the differences between different treatments. Pearson's *r* was used to assess the correlations between variables. A *p*-value of < 0.05 was regarded as significant for all of the tests.

3. Results and discussion

3.1. *General condition of the fish*

During the exposure period, the swimming and dietary behavior of fish were normal in all treatments. No death of fish occurred. None of the treatments significantly affected the whole body

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condition (data not shown). However, significant increases of KSI and SSI were found in 100 μg g^{-1} As(III) and As(V) treatments (Figure S1). Spleen enlargement is often associated with infections, while larger kidney are generally considered as an indication of chronic or high levels of pollution (Schmitt and Geological Survey (U.S.), 2002). The organ indexes were not affected by 50 μg g^{-1} As(III) or As(V) treatments, indicating that the fish were not under any metabolic stress.

3.2. *Arsenic bioaccumulation*

Table 1 presents the total As concentrations in different tissues of the fish after 10 d and 20 d dietborne exposure. After 50 and 100 μg g^{-1} As(III) and As(V) dietary exposure, significant accumulation of As was found in all the tissues compared with the control. After 20 days of exposure, As concentrations in muscles were not significantly different between As(V)and As(III) treatments. This indicated that the capacity of muscle to accumulate As did not depend on the iAs speciation at the end of exposure. However, in intestine, kidney, spleen and liver tissues, As concentrations were lower in As(III) treatments than in As(V) treatments after 20 d exposure. In particular, the As concentration in kidney was about 3 times lower in 100 μg g^{-1} As(III) treatment than 100 μg g^{-1} As(V) treatment. This suggested that As(V) possibly had greater ability than As(III) to pass through the intestinal wall, distributed via the circulatory system, and accumulated into fish tissues. Whaleymartin et al. (2012) found that As(III) were sequestered in the digestive gland due to binding with thiol moieties of proteins, while this sequestration could not found with As(V). However, a recent study showed controversial results with us that As(III) was more bioavailable than As(V) in Bombay oyster (*Saccostrea cucullata)* (Zhang et al., 2015). In addition, no significant differences was found with total As accumulations in muscle, liver or intestine between As(V) and As(III) treatments in marine fish (*Siganus fuscescens)* (Zhang et al., 2016a). Clearly,

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the bioavailability of iAs was species-specific. Other possible sources of variability for the observed results include the exposure pathway, exposure time and the differences in biotransformation, metabolism and distribution of As species, effect of different nutrients on iAs absorption.

In 100 μg g^{-1} As(V) treatment, the total As concentrations in all tissues excluding liver were lower at 20 d than those at 10 d (Table 1). The decreased As concentrations indicated that acclimation strategies occurred to regulate the body As levels. Similar results were reported previously using waterborne exposure. Allen et al. (2004) found that As accumulations in the muscles, kidney, liver, and gills in freshwater fish *Channa punctatus* reduced significantly after longer exposure (90 d) compared with 60 d after exposure to 13.4 mmol L^{-1} of arsenic trioxide. The acclimation was also found in rainbow trout *Salmo gairdneri* and in medaka *Oryzias mekongensis* exposed to iAs (Chen et al., 2018; Dixon and Sprague, 2010). Previous studies found three families of transporters aiding in decreasing cellular As concentrations in fish, including phosphate transporters, aquaglyceroporins (AQP) and multidrug resistance-associated proteins (MRP). It is considered that that As(V) is taken up via the phosphate transporters (Beene et al., 2011), while the uptake and translocation of As(III) are mediated by AQP (Jung et al., 2015). Multidrug resistance-associated proteins (MRP) are known to transport a series of anionic substrates including As-glutathione complex out of cells (Shaw et al., 2010). Jung et al. (2015) identified a novel variant of AQP3 in *Fundulus heteroclitus* intestine functioning in As transport and attributed its low bioaccumulation and high resistance to environmental As. Shaw et al. (2010) found that the MRP2 expression in the apical membrane of proximal tubules increased when *Fundulus heteroclitus* were exposed (waterborne) to As(III) for 4-14 d; therefore, the increased As elimination could explain the increased resistance to As in fish. The association between the

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toxicokinetic process of dietborne As and the influx/efflux transporters needs to be studied further.

3.3. *Biotransformation of arsenic*

3.3.1. Biotransformation between As(III) and As(V)

In As(V) treatments, As(III) was found in all tissues, suggesting that the reduction of As(V) to As(III) occurred in fish body (Figure 1 and 2, Table S2 and S3). However, the capability of As(V) reduction to As(III) was associated with exposure duration and As(V) dose. The As(V) reduction was identified as a detoxification process by reacting with glutathione enzymatically or nonenzymatically (Mrak et al., 2008; Shiomi et al., 1996). Although As(III) is generally more toxic than As(V), various processes could be initiated to cope with the toxicity of As(III) in organisms, including expelling As out of cells, transformation into organic As, and sequestration of intracellular As(III) by metal binding peptides, *e.g.*, metallothione-like protein (MTLP), phytochelatin (PC) and GSH (Allen and Rana, 2004; Clemente et al., 2019; Morelli et al., 2005).

In As(III) treatments, the As(V) was found in intestine, liver, kidney, spleen and muscle, and the concentration of As(III) in kidney is below the detection limit (3 μg kg^{-1}), indicating that As(III) could be oxidized to As(V) in crucian carp (Figure 1 and 2). The As(V) concentration and proportion increased by 2 ~ 7 folds in liver and muscle from 10 d to 20 d after exposure to As(III). It indicated that more As(III) was oxidized to As(V). Such result of As(III) oxidation to As(V) was previously reported in microorganisms but seldom reported in fish. The mechanisms of As(III) oxidation by microorganisms include extracellular oxidation of As(III) via an arsenite oxidase and chemolithoautotrophic metabolism where As(III) is utilized as an energy source (Rahman and Hassler, 2014). However, the mechanisms of As(III) oxidation in fish are not clear and need further exploration.

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Muscle is the main edible part of crucian carp. Since iAs species is far more toxic than organic As species, the accumulation of iAs species in fish muscle receive significant attention in risk assessment for human diet (Wolle et al., 2019). In 50 μg g^{-1} As(V) treatment, 1.257 ± 0.406 μg/g of As(III) was found in muscle at 10d; this was much higher than the As(III) found in As(III) treatment (Figure 1). The As(III) accumulation in muscle in As(V) treatment was not "persistent" when the exposure concentration is low (50 μg/g); the concentration and proportion of As(III) were significantly lowered at 20 d than 10 d (Figure 1). This indicated that fish could regulate their body As(III) levels by controlling the metabolic process. Mechanisms such as enzymatic induction or expression of As transporters may be the reason for the related regulation (Chen et al., 2018). When the exposure concentration of As was 100 μg g^{-1}, the concentration and proportion of As(III) in muscle were significantly higher in As(V) treatment than As(III) treatment, while the concentration and proportion of As(V) in muscle were significantly higher in As(III) treatment than As(V) treatment (Figure 2, Table S3), after 20 days of exposure. Considering the higher toxicity of As(III) than As(V) for human diet, such parallel conversion between As(III) and As(V) in crucian carp should attract much attention for assessing the health risk of aquatic iAs (Wolle et al., 2019).

3.3.2. Biotransformation of inorganic arsenic to methylated species.

To reflect the biomethylation process of iAs precisely, two indices were introduced including the primary methylation index [PMI = (MMA + DMA)/total As] and the secondary methylation index [SMI = DMA/(MMA + DMA)] to evaluate the methylation ability of iAs-exposed individuals (Li et al., 2011). From the results in Table 2, Figure 1 and Figure 2, the levels of methylation indexes and the concentrations of MMA and DMA in intestine were even higher than in the liver for certain treatments, which indicated a high methylation activity in the intestine or gut contents. Methylation of iAs by intestinal cells has been previously reported (Calatayud et al.,

Journal Pre-proof

2012). In addition, as an external ingestion part, the intestine might be affected by intestinal microorganisms and surrounding environment. In 100 μg g^{-1} As(V) treatments, the SMI in spleen and kidney increased significantly over time, suggesting enhanced secondary methylation process (Table 2). In 100 μg g^{-1} As(III) treatments, however, the PMI in liver and the SMI in kidney decreased significantly, indicating reduced methylation process. The decreased PMI was likely due to that the methylation ability of liver was saturated by high iAs loads, since the iAs fraction increased from 22.1% at 10 d to 47.7% at 20 d. The rest iAs that cannot be methylated may release into blood and subsequently other organs and tissues (Lewchalermvong et al., 2018). Similarly, the elevated levels of MMA in kidney may be responsible for the decreased SMI in kidney due to the inhibition or saturation of dimethylation after prolonged exposure to As(III). The compromised methylation upon prolonged As(III)-exposure was also reported previously in rat, which was attributed to the diminished methyltransferase activity and/or consumptive depletion of the methyl donor *S*-adenosylmethionine (Csanaky et al., 2003). Impaired methylation may lead to the retention of As(III) in body, as except for excretion in GSH-conjugated form, methylation is the other course responsible for As(III) elimination (Buchet and Lauwerys, 1985). The different capacity of methylation of As(III) or As(V) in fish found in this study have significant implications for iAs toxicity, given that As methylation ability is correlated with As-related diseases (Mrak et al., 2008; Shiomi et al., 1996).

3.3.3. Biotransformation to AsB and AsC.

After 20 d of exposure, AsB was the predominant As species in muscle, liver, kidney, and spleen (Table S2 and S3), suggesting that crucian carp can convert MMA and DMA to AsB, which is one of the less reactive and toxic As species. It has been found that AsB accounts for more than 95% of arsenicals found in marine fish (Francesconi and Edmonds, 1996). However, in freshwater

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fish medaka (*Oryzias latipes*) and tilapia (*Tilapia mossambica*), MMA and trimethylarsenate were found as the predominant As species after waterborne As exposure (Suhendrayatna et al., 2002a; Suhendrayatna et al., 2002b). Our present study is among the few studying the dietary As exposure in freshwater fish.

It was reported that, AsB could be biosynthesized through iAs methylation and subsequently transformation. Thomas et al. (2004) reported that As(V) is first reduced into As(III) and then converted to MMA, DMA and trimethylated products. Edmonds (2000) and Ritchie et al. (2004) proposed that dimethylarsenoriboses could be degraded to dimethylarsinylethanol and then converted to either AsC or dimethylarsinylacetic acid, further oxidation of the primary alcohol group of AsC results in the formation of AsB. However, only less than 3.5% of AsC was detected in intestine and liver of fish, and no AsC was found in other tissues in our study, suggesting oxidation of AsC is not the pathway for the formation of AsB in crucian carp. Another possible pathway is that DMA could replace ammonium ions in the synthesis of amino acids; therefore, 'arsenylation' of pyruvate, paralleling the biosynthesis of glyoxylate, or alanine could give rise to the synthesis of AsB (Edmonds, 2000). No conclusion has been made on formation mechanism of AsB in fish, further research is thus needed to supplement the profile of iAs biotransformation.

The As biotransformation and distribution in fish can be influenced by the metabolic role of tissues and the different As assimilation-elimination relationships between them. Intestine is the region for As absorption and was probably the first location of As biotransformation. Among all tissues, the largest proportion of methylated As (MMA and DMA) and the smallest proportion of AsB was found in intestine, which might be due to that it faced the dietborne iAs firstly and had low ability of transforming methylated forms to AsB (Zhang et al., 2016a). Liver is considered as the most important organ responsible for As biotransformation (Wood, 2011). The complex As

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forms absorbed through the intestine could be further transformed in liver before translocated to other tissues or excreted via bile. The fraction of AsB in liver was therefore found much higher than that in intestine. Kidney is known to be important in elimination processes (Wood, 2011). It accumulated the most As among tissues and AsB was the predominant As species. Therefore, it is likely that the diet iAs could be finally transformed to AsB and then eliminated out of fish body through kidney. In addition, high level of As, mainly AsB, was found accumulated in spleen. The largely accumulation of As was considered responsible for the spleen enlargement. Since spleen was one of the main immunocompetent organs in fish, the dietary iAs exposure could possibly have a profound influence on the fish immune function. Ghosh et al. (2006) reported that exposure to non-lethal concentrations of As induced a gross histological damage and the decline of T and B cell responses in spleen of catfish *Clarias batrachus*.

3.4. *Oxidative stress in organs*

Arsenic-induced excess production of reactive oxygen species (ROS) and oxidative stress have been implicated to play vital role in the aetiopathogenesis of As related diseases. As a by-product of unsaturated fatty acids peroxidation, MDA is a reliable indicator to estimate the extent of lipid peroxidation damage of cell membrane (Chen et al., 2019). Meanwhile, the levels of antioxidant substance GSH and antioxidant enzymes like SOD and CAT are appropriate indirect measurements to assess the antioxidant status in organs.

As shown in Figure 3 and 4, oxidative biomarkers showed different responses in organs of fish by iAs exposure. In liver of 100 μg g^{-1} As treatments, the GSH level was significantly upregulated by As(III) exposure and the CAT activity was enhanced by As(V) exposure. The MDA content significantly increased at 10 d, suggesting oxidative damage of the cell membrane. However, the MDA content decreased to the normal level at 20 d, suggesting the occurrence of a recovery

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mechanism. In kidney of 100 μg g^{-1} As treatments, the SOD and GSH levels increased upon As(III) exposure and the CAT activity increased upon As(V) exposure. No change of MDA level was found in kidney during the exposure to 100 μg g^{-1} As(V); however, the level of MDA in kidney increased by 4 fold upon 100 μg g^{-1} As(III) exposure, suggesting a serious oxidative damage. Similar to in liver, this damage was also not detected after 20 days. In spleen of 100 μg g^{-1} As treatments, however, oxidative damage was not be induced during the 20 d dietary exposure. These differences between organs might be due to different antioxidant strategies to achieve protection against As toxicity or the different As species reaching different organs. Additionally, 50 μg g^{-1} As(III) and As(V) dietborne exposure could not lead to oxidative damages in liver, kidney or spleen (Figure 4). There is evidence that iAs waterborne exposure lead to ROS generation and lipid peroxidation in fish. Nevertheless, with continuous exposure, antioxidant defense systems could be activated to decrease ROS and MDA generation (Kim and Kang, 2015; Sarkar et al., 2014). Our discovery of the reduced MDA level as well as the increased GSH levels demonstrated that crucian carp could activate commensurate defense systems to resist the oxidative stress caused by dietary iAs at environmentally relevant doses.

3.5. *Association between arsenic methylation and oxidative damage*

As shown in Figure 5, the MDA levels was found significantly and positively correlated with SMI in liver in 100 μg g^{-1} As(V) treatment and kidney in 100 μg g^{-1} As(V) treatment and 100 μg g^{-1} As(III)-10d-treatment. This suggested that dimethylation was probably responsible for the oxidation damage in organs. In liver of fish by As(V) exposure, the slope of regression line on 10 d was found greater than that on 20 d, indicating that liver could be deeper poisoned on 10 d by dimethylation process before antioxidant system could give adequate response to eliminate ROS. In kidney of 100 μg g^{-1} As(III) treatment, the SMI after 20 d was significantly decreased (> 2 times)

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than 10 d (Table 2). Apart from antioxidative systems, the reduced dimethylation might also work to decrease ROS generation originated from dimethylation process (Lu et al., 2011). In our study, there were no apparent associations between organs MDA activities and levels of As(V), As(III), iAs, MMA(or MMA%), DMA(or DMA%),total As, and PMI in each of exposure group (figures not shown). Possibly, different As species have different pharmacokinetic properties. SMI calculated as DMA/(MMA + DMA) was manifested a more consistent indicator of As bioconversion capacity than other ratios or species levels (concentrations or proportions), probably because both DMA and MMA have similar biological half-lives in liver and kidney of crucian carp. In addition, SMI calculated as DMA/MMA, which were developed for arsenic methylation capacity previously (Chen et al., 2003; Tseng et al., 2005), however, did not show apparent correlation with MDA level in our study (figures not shown). This might due to that, according to the path of iAs biotransformation, secondary methylation could only conduct on the basis of primary methylation.

Previously, some epidemiological studies showed that poor efficiency of second methylation indicated by low percent of DMA or low DMA/MMA ratio in urine is connected with increased risk of arsenic-related diseases (Huang et al., 2009; Pu et al., 2007). These results were attributed to the detoxification of second methylation by author, *i.e.*, fully methylated iAs to DMA is needed to minimize As toxicity. This basically coincided with the previous studies and suggested that poor second methylation could result in accumulation of trivalent MMA, which showed the toxicity equal to or greater than As(III) in cytotoxicity studies (Petrick et al., 2000). Contradictorily, other epidemiological studies showed the positive association between higher DMA% or secondary methylation index (SMI) in urine and increased risk of arsenic-related diseases including urothelial cancer, obesity, diabetes and the metabolic syndrome (Kuo et al., 2015; Melak et al., 2014; Nizam

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et al., 2013). It has been demonstrated that, dimethylation could be a bioactive procedure where DMA(V) could convert to DMA(III), a highly toxic dimethylated arsenic species (Lu et al., 2011). Douillet et al. (2013) reported that the inhibition of glucose-stimulated insulin secretion in islets exposed to As(III) or MMA(III) was possibly due to the formation of DMA(III). Furthermore, this inhibition was reported probably attributed to the provoked ROS levels and elevated oxidative stress (Fu et al., 2010). Rehman et al. (2014) demonstrated that methylated biotransformation caused apoptosis of leukemia HL-60 cells, while DMA(III) was a more potent inducers of ROS generation than MMA(III) and As(III). Particularly, DMA(III) has been indicated to interwork with the voltage-dependent anion channel, which induced the release of citochrome c from mitochondria (Rehman et al., 2014).

Over the years, whether the methylation of As is to detoxify or potentiate arsenic toxicity still remains an ongoing debate. The association between SMI and lipid peroxidation damage found in our present research was scarcely reported before as experimental evidence from animal studies. In the future, the specific transformation and pharmacokinetic mechanism of arsenicals in different organisms are needed to provide useful information on the profile of iAs metabolism. Of these, a complete examination of the 'metabolic behavior of dimethylation process' needs to be a priority, given the unique association between SMI and MDA depicted in our research. In addition, we could not dispute that thorough methylation (and/or further organification) may be a detoxication terminus for fish. It could be speculated that, from our research, the intensity and duration of the toxic action is proportional to the dimethylation rate and the length of time there remaining, respectively. Maybe, alterations in the rate of As dimethylation could have vital implications for assessing the threat derived from exposure to this metalloid.

4. Conclusions

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This study for the first investigated the biotransformation of dietary inorganic As and the association between As methylation and lipid peroxidation damage in fish. The capabilities of crucian carp to accumulate and transform iAs greatly varied depending on the exposure iAs species, exposure dose, exposure duration, and test tissue. Particularly, the parallel conversion between As(V) and As(III) in muscle implies the changeable toxicity of iAs by crucian carp before consumed by human. Arsenic dietary exposure could provoke oxidative stress, the level of which highly depends on the exposure iAs species and test tissues. The oxidative damage induced after 10 d dietborne exposure was subsequently removed after 20 d. Noteworthy, the damage was demonstrated to be associated with dimethylation activity of arsenic. The research provides useful information for clarifying the uncertainty in the toxicity evaluation for iAs metabolism.

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Supporting information

As species concentrations (μg g^{-1}, dry weight) and proportions (%) in control (basal) and As-dosed diets. Fractions of As species (%) in intestine, liver, kidney, spleen, and muscle tissues of crucian carp (*Carassius auratus*) after 50 μg g^{-1} of As(III) and As(V) dietborne exposure for 10 d and 20 d. Fractions of As species (%) in intestine, liver, kidney, spleen, and muscle tissues of crucian carp after 100 μg g^{-1} of As(III) and As(V) dietborne exposure for 10 d and 20 d. Liver somatic index (A), kidney somatic index (B), and spleen somatic index (C) in crucian carp after 50 μg g^{-1} of As(III) and As(V) dietborne exposure for 10 d and 20 d. Liver somatic index (D), kidney somatic index (E), and spleen somatic index (F) in crucian carp after 100 μg g^{-1} of As(III) and As(V) dietborne exposure for 10 d and 20 d.

Author contributions

Di Cui: Conceptualization, Methodology, Validation, Investigation, Supervision, Project administration, Data Curation, Writing - Original Draft, Writing - Review & Editing.

Peng Zhang: Writing - Review & Editing, Validation, Funding acquisition, Formal analysis, Visualization, Software.

Haipu Li: Resources, Supervision, Funding acquisition, Writing - Review & Editing.

Zhaoxue Zhang: Methodology, Writing - Review & Editing, Data Curation.

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Figure Captions

Figure 1. As species concentrations in intestine, liver, kidney, spleen, and muscle tissues of crucian carp after 50 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d.

Figure 2. As species concentrations in intestine, liver, kidney, spleen, and muscle tissues of crucian carp after 100 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d.

Figure 3. SOD, CAT activities and GSH, MDA contents in liver, kidney, and spleen of crucian carp after 50 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d.

Figure 4. SOD, CAT activities and GSH, MDA contents in liver, kidney, and spleen of crucian carp after 100 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d.

Figure 5. Correlation between secondary methylation index (SMI), which was calculated as DMA/(MMA + DMA), and malondialdehyde (MDA) in liver (A) and kidney (B) of crucian carp after 100 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d.

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Table 1. Total As concentrations in liver, kidney, spleen, intestine and muscle tissues of crucian carp (*Carassius auratus*) after 50 μg g^{-1} and 100 μg g^{-1} of As(III) and As(V) dietborne exposure for 10 d and 20 d. All values are means ± SD (n = 7). Different letters indicate significant differences among treatments of the same exposure dose (p<0.05). BDL (below detection limit); As(III), arsenite; As(V), arsenate

	Total As concentrations in tissues (μg g^{-1})				
	Intestine	Liver	Kidney	Spleen	Muscle
50 μg g^{-1}					
control	0.033±0.03 ^{a}	0.02±0.00 ^{a}	0.00±0.00 ^{a}	0.01±0.00 ^{a}	0.16±0.02 ^{a}
As(III)-10d	0.80±0.07 ^{b}	0.45±0.12 ^{b}	1.88±0.34 ^{b}	0.53±0.08 ^{b}	0.35±0.10 ^{b}
As(III)-20d	0.83±0.06 ^{b}	0.58±0.10 ^{b}	4.39±1.65 ^{c}	0.72±0.11 ^{b}	0.37±0.07 ^{b}
As(V)-10d	0.72±0.05 ^{b}	0.51±0.12 ^{b}	3.86±0.51 ^{c}	0.52±0.10 ^{b}	1.54±0.29 ^{c}
As(V)-20d	1.00±0.07 ^{b}	0.64±0.11 ^{b}	7.46±1.12 ^{d}	1.15±0.24 ^{c}	0.43±0.11 ^{b}
100 μg g^{-1}					
control	0.033±0.03 ^{a}	0.02±0.00 ^{a}	0.00±0.00 ^{a}	0.01±0.00 ^{a}	0.16±0.02 ^{a}
As(III)-10d	2.51±0.29 ^{c}	1.59±0.31 ^{b}	4.01±0.73 ^{c}	4.38±0.66 ^{c}	1.22±0.22 ^{c}
As(III)-20d	2.20±0.41 ^{b}	1.64±0.22 ^{b}	2.78±0.28 ^{b}	2.38±0.34 ^{b}	0.90±0.27 ^{b}
As(V)-10d	3.87±0.53 ^{d}	2.95±0.46 ^{d}	12.16±2.00 ^{c}	6.06±0.84 ^{d}	1.29±0.08 ^{c}
As(V)-20d	2.46±0.39 ^{c}	2.42±0.32 ^{c}	7.55±0.81 ^{d}	4.83±0.59 ^{c}	1.12±0.24 ^{b}

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Table 2. The primary methylation index (PMI) and secondary methylation index (SMI) in intestine, liver, kidney, and spleen of crucian carp after 50 μg g^{-1} and 100 μg g^{-1} of As(III) and As(V) dietborne exposure for 10 d and 20 d. All values are means ± SD (n = 7). Different letters indicate significant variation between different periods of exposure. (p<0.05).

	Intestine		**Liver**		**Kidney**		**Spleen**	
	PMI	SMI	PMI	SMI	PMI	SMI	PMI	SMI
50 μg g^{-1}								
As(III)-10d	0.52±0.08	0.58±0.08	0.38±0.09	0.45±0.09	-	-	-	-
As(III)-20d	0.58±0.09	0.60±0.09	0.36±0.13	0.37±0.08	-	-	-	-
As(V)-10d	0.43±0.03 ^{a}	0.61±0.09	0.24±0.05	0.33±0.08	0.12±0.03	0.30±0.09	-	-
As(V)-20d	0.53±0.03 ^{b}	0.68±0.11	0.29±0.08	0.49±0.12	0.08±0.02	0.33±0.12	-	-
100 μg g^{-1}								
As(III)-10d	0.43±0.06 ^{a}	0.41±0.08	0.29±0.04 ^{b}	0.38±0.08	0.05±0.00	0.61±0.09 ^{b}	0.08±0.01	0.39±0.04
As(III)-20d	0.52±0.07 ^{b}	0.32±0.08	0.21±0.03 ^{a}	0.36±0.11	0.06±0.00	0.28±0.04 ^{a}	0.07±0.01	0.40±0.03
As(V)-10d	0.54±0.08 ^{b}	0.18±0.02 ^{a}	0.17±0.03	0.36±0.06	0.12±0.03	0.23±0.04 ^{a}	0.11±0.01	0.47±0.08 ^{a}
As(V)-20d	0.38±0.02 ^{a}	0.43±0.06 ^{b}	0.23±0.04	0.40±0.08	0.09±0.02	0.36±0.07 ^{b}	0.09±0.01	0.69±0.11 ^{b}

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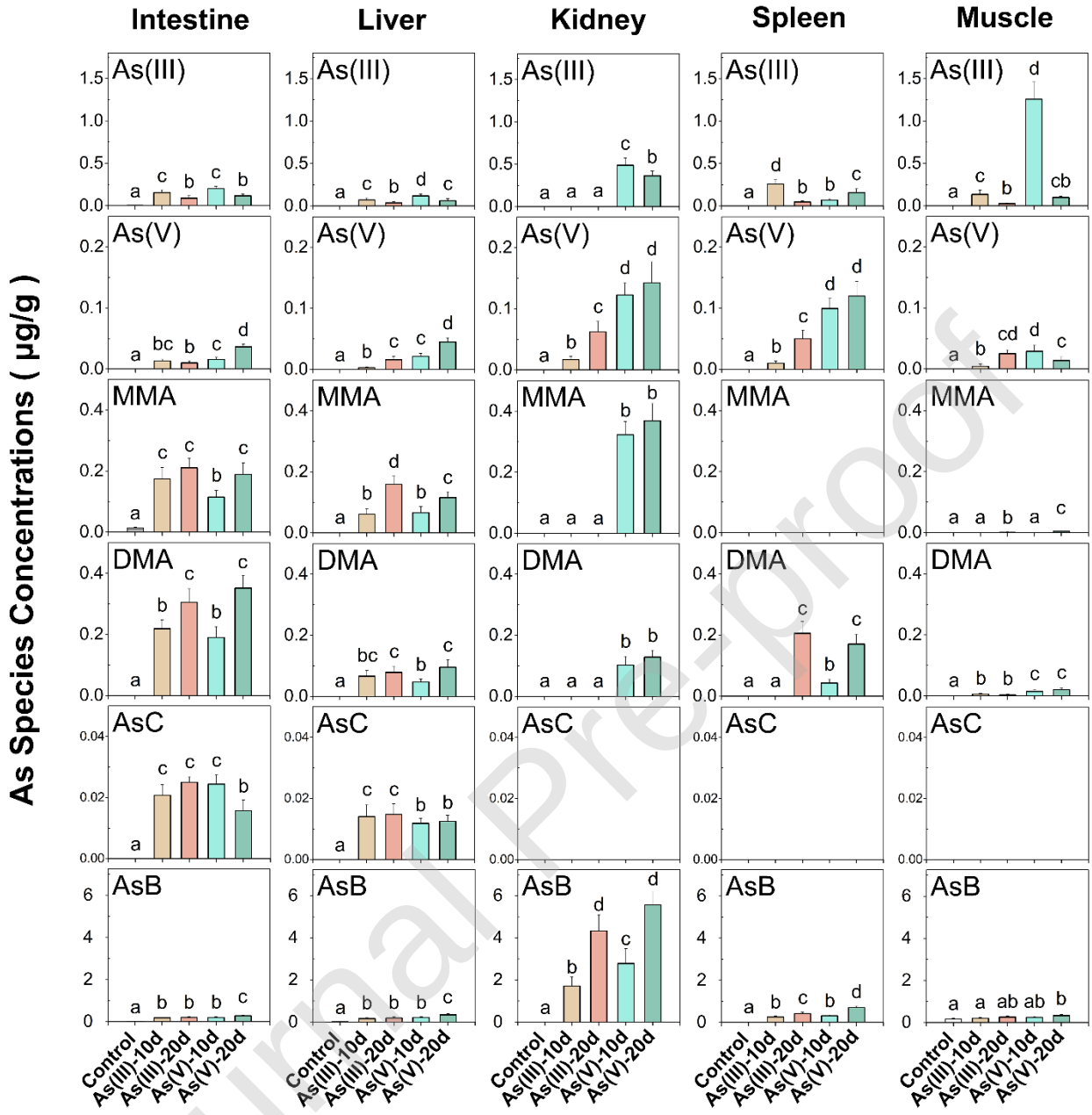

Figure 1. As species concentrations in intestine, liver, kidney, spleen, and muscle tissues of crucian carp after 50 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d. Values are means ± SD (n = 7). Different letters indicate significant differences among treatments (p<0.05). As(V), arsenate; As(III), arsenite; MMA, monomethylarsonate; DMA, dimethylarsinate; AsC, arsenocholine; AsB, arsenobetaine.

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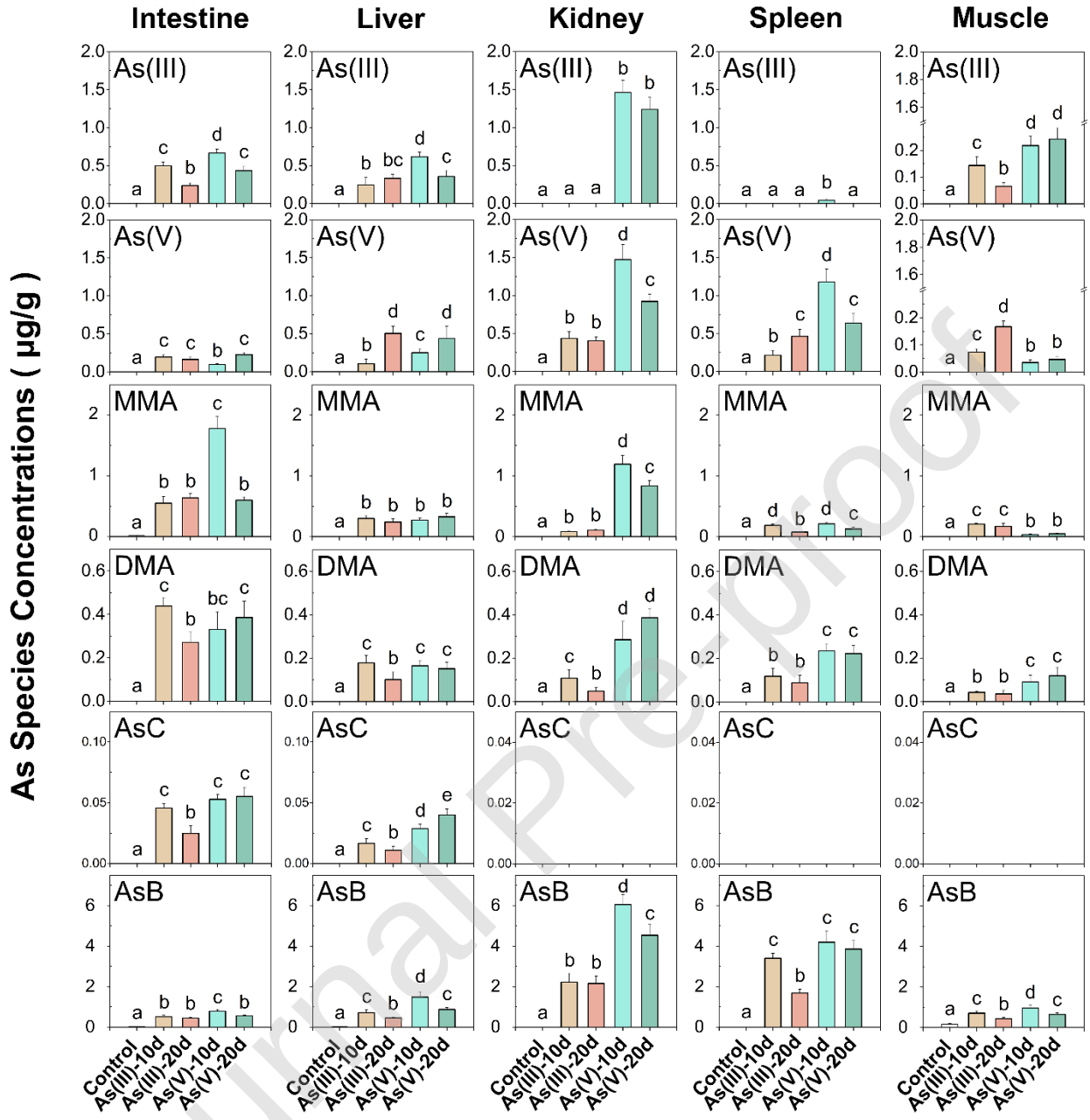

Figure 2. As species concentrations in intestine, liver, kidney, spleen, and muscle tissues of crucian carp after 100 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d. Values are means ± SD (n = 7). Different letters indicate significant differences among treatments (p<0.05).

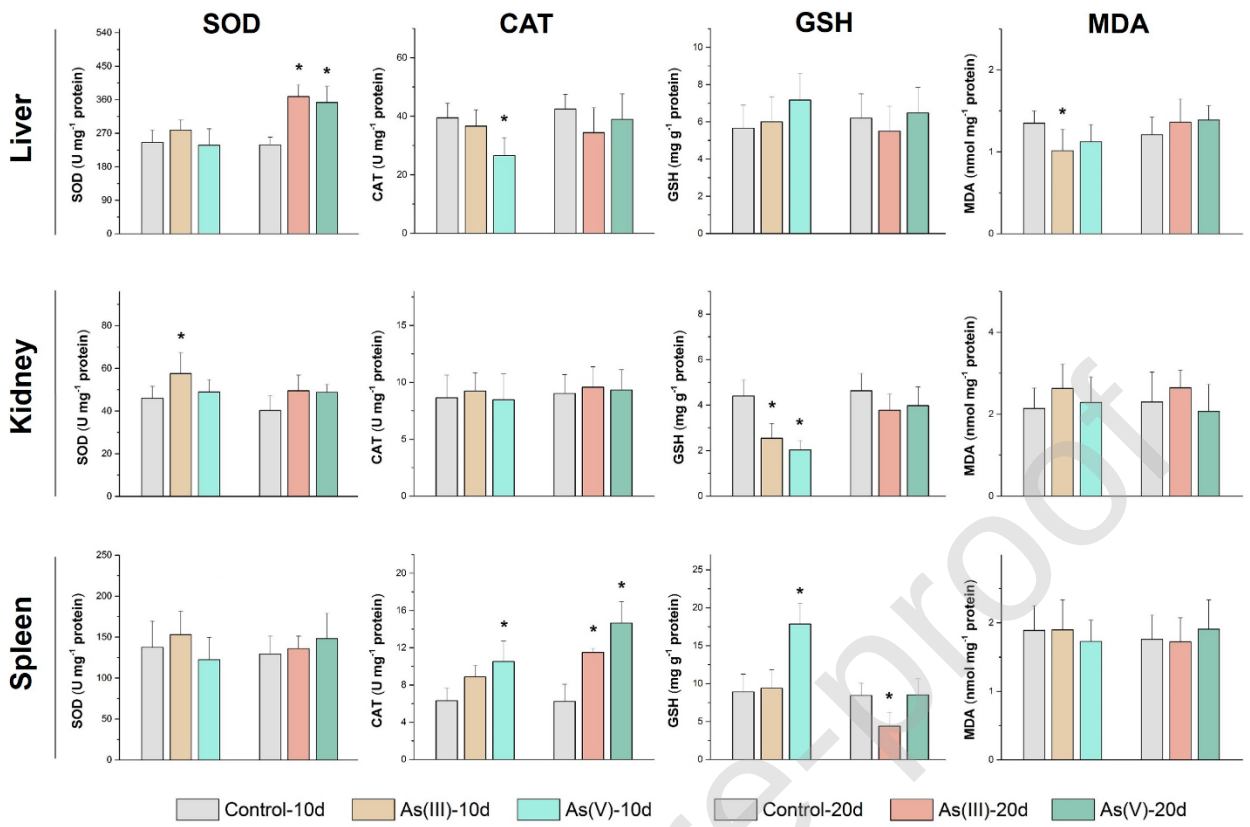

Figure 3. SOD, CAT activities and GSH, MDA contents in liver, kidney, and spleen of crucian carp after 50 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d. Values are means ± SD (n = 7). Significant differences versus control are marked with * (p<0.05).

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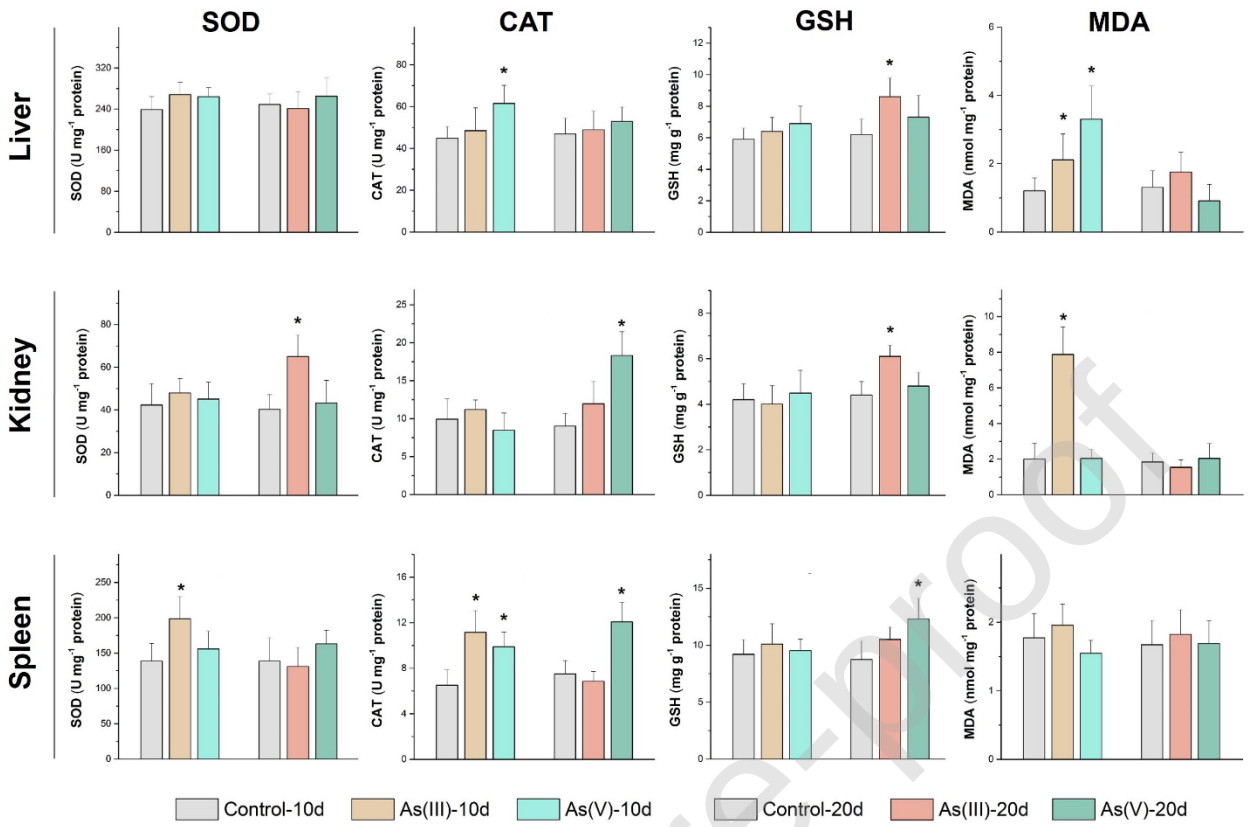

Figure 4. SOD, CAT activities and GSH, MDA contents in liver, kidney, and spleen of crucian carp after 100 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d. Values are means ± SD (n = 7). Significant differences versus control are marked with * (p<0.05).

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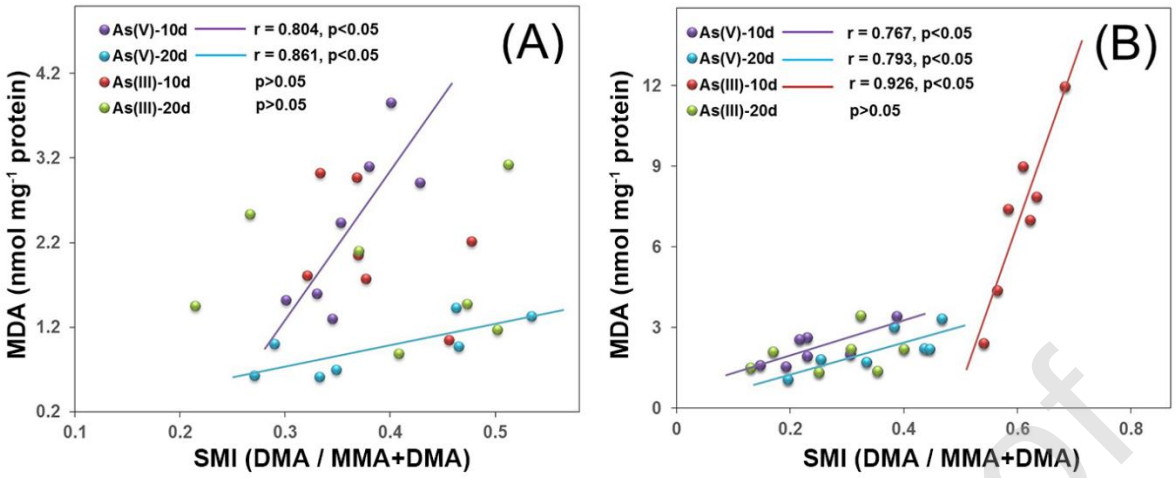

Figure 5. Correlation between secondary methylation index (SMI), which was calculated as DMA/(MMA + DMA), and malondialdehyde (MDA) in liver (A) and kidney (B) of crucian carp after 100 μg g^{-1} As(III) and As(V) dietborne exposure for 10 d and 20 d.

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